Properties of voltage-gated chloride channels of the ClC gene family

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We review the properties of ClC chloride channels, members of an expanding gene family originally discovered by the cloning of the ClC-0 chloride channel from *Torpedo* electric organ. There are at least nine different ClC genes in mammals, several of which seem to be expressed ubiquitously, while others are expressed in a highly specific manner (e.g. the muscle-specific ClC-1 channel and the kidney-specific ClC-K channels). The newly cloned rat ClC-4 is strongly expressed in liver and brain, but also in heart, muscle, kidney and spleen. ClC chloride channels are structurally unrelated to other channel proteins and have twelve putative transmembrane domains. They function as multimers with probably four subunits. Functional characterization is most advanced with ClC-0, ClC-1 (mutations which cause myotonia) and ClC-2, a swelling-activated chloride channel. Many of the new ClC family members cannot yet be expressed functionally.

A couple of years ago, the expression cloning (Jentsch *et al.* 1990) of the voltage-gated chloride channel ClC-0 provided an entry point into a new, presumably large, gene family of chloride channels. At this point in time, we know more than eight different members (genes) of this family in a single mammalian species, only some of which are published so far (Fig. 1*A*).

The family can be subdivided into different branches, as visualized by the dendrogram in Fig. 1*B*. The major skeletal muscle chloride channel ClC-1 (Steinmeyer *et al.* 1991*a*) and the ubiquitous swelling-activated Cl^--channel ClC-2 (Gründer *et al.* 1992; Thiemann *et al.* 1992), which have an overall identity of roughly 50%, belong to one of these branches. Though belonging to a different species, the *Torpedo* electric organ channel ClC-0 is shown for completeness. As expected, it is most related to ClC-1, since the electric organ is derived developmentally from skeletal muscle; in contrast to ClC-1, ClC-0 is also expressed to an appreciable degree in (*Torpedo*) brain. The kidney-specific channels ClC-K1 and ClC-K2 (Uchida *et al.* 1993; Adachi *et al.* 1994; Kieferle *et al.* 1994) (and, of course, their human counterparts hClC-Ka and -Kb (Kieferle *et al.* 1994)) are distant cousins in this branch. They are highly homologous to each other (about 80–90%), but are differentially distributed along the nephron. A more distant branch of this family is represented by ClC-3 (cloned from rat, Kawasaki *et al.* 1994) and ClC-4 (human, van Slegtenhorst *et al.* 1994). We have independently cloned rat ClC-4 from brain using a RT-PCR approach. Both rat sequences are aligned in Fig. 2*B*. They are about 78% identical, but show less than 30% identity to the other sub-branch in this gene family. While rat ClC-3 is rather ubiquitously expressed (Kawasaki *et al.* 1994), ClC-4 expression is more restricted at least in humans (van Slegtenhorst *et al.* 1994). With human ClC-4, expression is highest in muscle and brain, while expression of rat ClC-4 is most prominent in liver and brain (Fig. 2*A*). Additional expression is found in heart, muscle, spleen and kidney, while lung and testes are negative by Northern analysis. The smaller band seen in spleen is still compatible with the length of the open reading frame and could be due to a difference in splicing or polyadenylation (Fig. 2*A*). The function of these putative channels is not yet known, especially since we were unable to obtain functional expression as chloride channels (but see Kawasaki *et al.* (1994) for ClC-3).

Structural properties of ClC proteins

The transmembrane topology of ClC chloride channels is still largely conjectural, since it is mostly based on hydropathy analysis. This analysis, when applied to the first member of this gene family, ClC-0, led to the prediction of 12–13 transmembrane spans (Jentsch *et al.* 1990). However, it was noted that several putative transmembrane spans were not very hydrophobic, and some putative transmembrane domains are not clearly interrupted by hydrophilic stretches (especially in the D9–D12 region). D13 is a domain of intermediate hydrophobicity separated from the hydrophobic backbone of the channel by a large hydrophilic segment. We now know that this domain is highly conserved among different members of the ClC superfamily, but that it is probably cytoplasmic. This conclusion has been obtained from transplantation experiments, where a N-terminal domain important for the swelling-activation of ClC-2 was inserted before or after D13 (Gründer *et al.* 1992). This domain was functional in both positions. As it is very likely that its function requires binding to the cytoplasmic aspect of the channel backbone, D13 probably does not cross the membrane (Gründer *et al.* 1992). Another piece of information came from studies on glycosylation (Kieferle *et*

A

Voltage-gated chloride channels of the ClC family

	Tissue distribution	Function	Voltage dependence	Pore	Structure
ClC-0	Torpedo electric organ, skeletal muscle, brain	Stabilization of V in electric organ and muscle	Slow gate opened by hyperpolarization fast gate opened by depolarization	Linear 10 pS, double-barrel Cl > Br, I block	~12 hydrophobic transmembrane domains (TMD)
ClC-1	Mammals skeletal muscle (smooth muscle, heart)	Stabilization of V in skeletal muscle defect: myotonia	Deactivates with hyperpolarization inward rectifier in positive V range	~1 pS Cl > Br > I I block	~12 TMDs ≈ tetramer
ClC-2	Mammals ubiquitous	Cell volume regulation	Slowly activates with hyperpolarization closed at resting V linear once activated	~3–5 pS Cl ≥ Br > I I block	id.
ClC-3	Mammals broad distribution	?	? or outward rectifier	? pS (I > Cl = Br ?)	id.
ClC-4	Mammals rather broad	?	?	?	id.
ClC-K1	Mammalian kidney (more restricted than -K2)	Cl reabsorption ?	? or outward rectifier	? pS (Br > Cl > I ?)	id.
ClC-K2	Mammalian kidney	Cl reabsorption ?	? or outward rectifier	? pS (Br > I > Cl ?)	id.

B

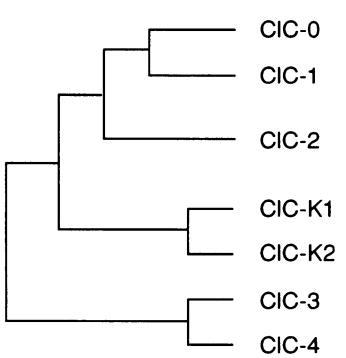

Fig. 1. *A*, summary of published ClC proteins and some of their characteristics. *B*, dendrogram showing the relationship between different members of the ClC family. Except for ClC-0, all proteins are from rat. The dendrogram has been obtained by the Pileup program of the software package of the University of Wisconsin Genetics Group.

al. 1994). The cDNAs of all ClC proteins known so far have one or more potential sites for N-linked glycosylation in the segment linking D8 and D9. This segment, however, was previously thought to be cytoplasmic. Using *in vitro* translation in the presence of pancreatic microsomes, Kieferle *et al.* (1994) have now shown that ClC-0, ClC-1, ClC-2 and ClC-K channels are glycosylated. By mutating the consensus site between D8 and D9 in the ClC-K channels, we could demonstrate that this site is indeed glycosylated. Glycosylation of the equivalent segment of ClC-0 was also demonstrated for the native channel isolated from *Torpedo* electric organ (Middleton *et al.* 1994).

This places the loop connecting D8 and D9 to the outside, and requires that either one putative transmembrane domain N-terminal of this segment does not cross the membrane, or, alternatively, that an additional transmembrane span is present. Since D4 is only weakly hydrophobic, and because it is not well conserved in newer members of this gene family, we propose that this domain does not cross the membrane (Fig. 3). On the other hand, Adachi *et al.* (1994) have reported a splice variant of ClC-K2 which entirely deletes D2 and surrounding conserved sequences. It was reported that this deletion mutant is functional and yields currents indistinguishable from wild-type ClC-K2. However, we think that this is unlikely, as D2 is very hydrophobic in the majority of ClC proteins (but less so with the ClC-Ks). To probe for the importance of D2 in an unambiguously expressible ClC channel, we deleted this segment in ClC-0 (S. Kieferle and T.J. Jentsch, unpublished). Its deletion destroyed channel activity, demonstrating that it is necessary at least in the context of ClC-0. This is compatible with its role as a transmembrane span, though it does not prove this point.

Placing the D8–D9 linker on the outside, while retaining an intracellular C-terminus (Gründer *et al.* 1992) also requires a revision of the D9–D12 segment, which is a broad hydrophobic region in all ClC proteins. We have now to envisage that the number of transmembrane crosses in this region is uneven (3 or 5). At present, we cannot distinguish between these possibilities. Evidently, much

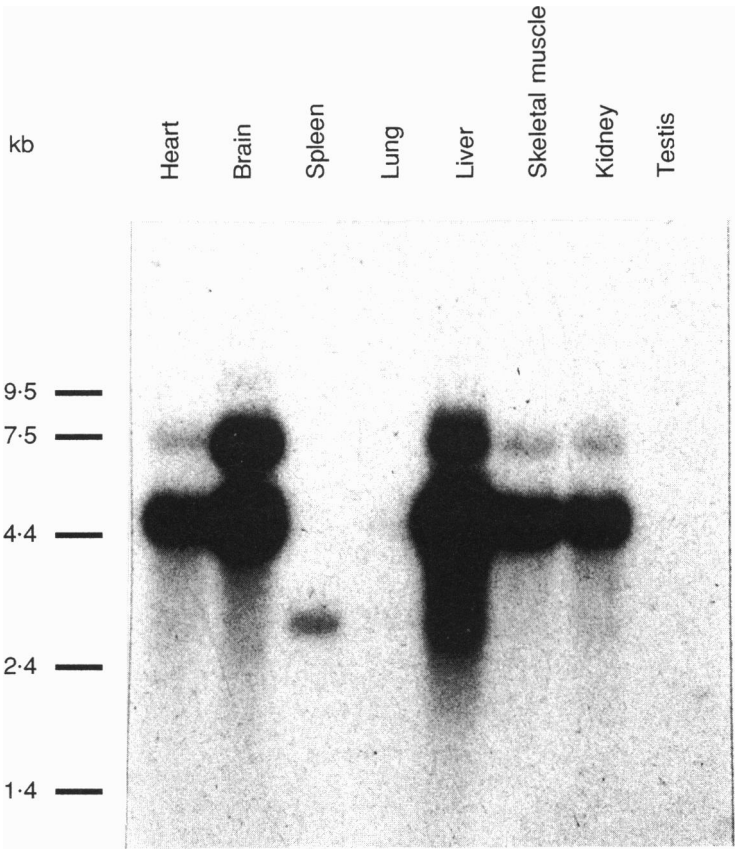

Fig. 2*A*, the ClC-3/ClC-4 sub-branch of the gene family; tissue distribution of rat ClC-4 by Northern analysis. A Northern blot containing rat mRNAs from several tissues was obtained from Clontech and probed under high stringency with a rat ClC-4 probe.

more work is needed to clarify ClC transmembrane topology. This information is direly needed for the interpretation of results from ongoing mutagenesis experiments.

Do ClC proteins form ion channels as monomers, or as (homo)multimers? The latter possibility was already suggested by the 'double-barrelled' nature of the ClC-0 *Torpedo* channel (Miller & White, 1984), and the finding that the single ClC-0 cDNA is sufficient for reproducing this feature (Bauer *et al.* 1991). The most conclusive information concerning the multimeric structure of ClC channels comes from a detailed functional analysis of two

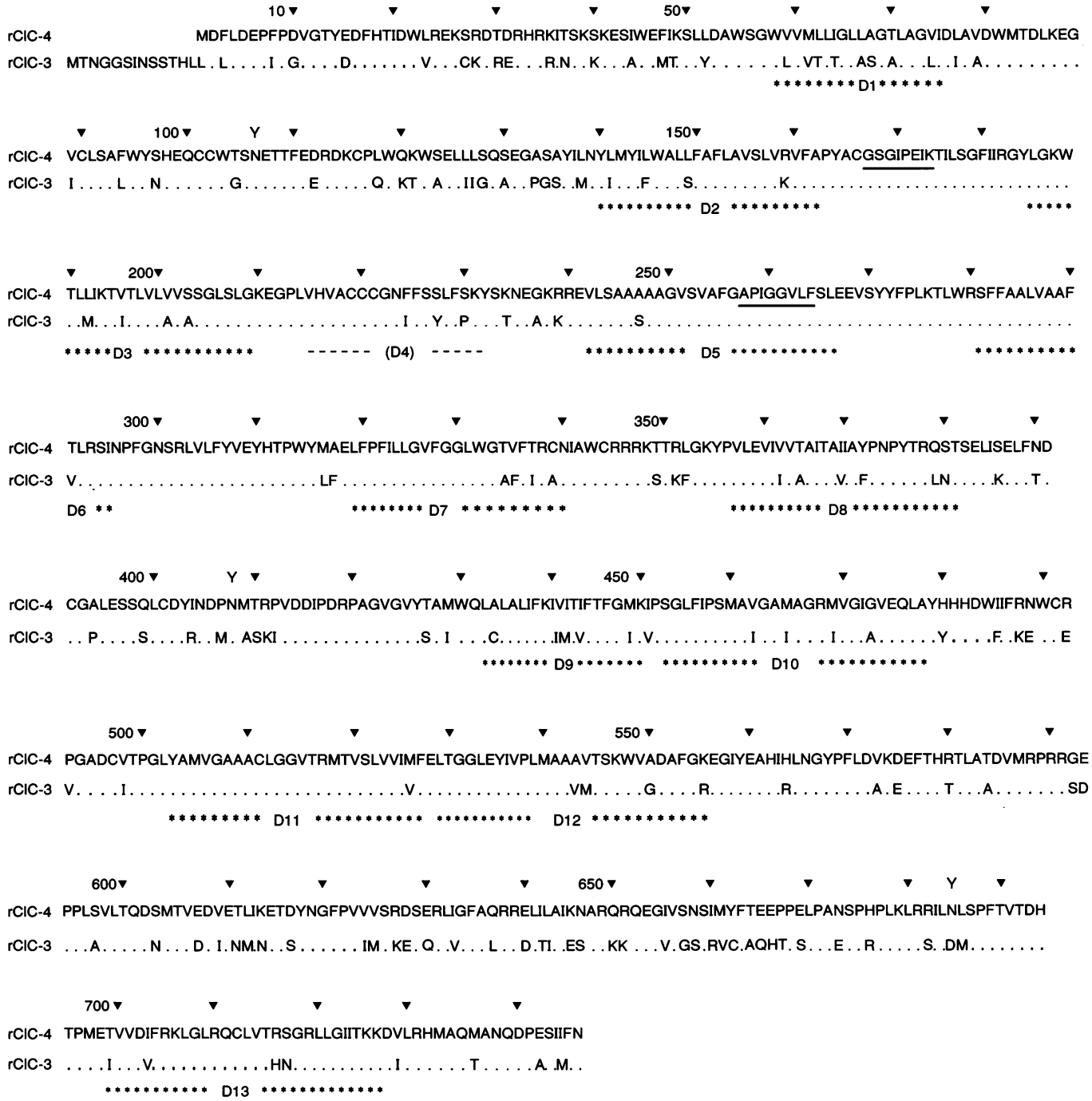

Fig. 2*B*, the ClC-3/ClC-4 sub-branch of the gene family; sequence alignment of ClC-4 with ClC-3. On top, the amino-acid sequence of rat ClC-4 is shown, and below the amino acids differing in rat ClC-3 (Kawasaki *et al.* 1994) are indicated. Domains D1 to D13 according to the original nomenclature (Jentsch *et al.* 1990) are shown, and the sequences used to generate primers to clone ClC-4 by RT-PCR from rat brain cDNA are underlined. Potential N-linked glycosylation sites are indicated by a Y above the sequence. The two proteins are 78% identical, and rat ClC-4 is 98% identical to human ClC-4 (van Slegtenhorst *et al.* 1994) at the amino acid level. The nucleic acid sequence of rat ClC-4 has been deposited in the EMBL/GenBank database, accession number Z36944.

human mutations in the muscle channel ClC-1 which lead to dominant myotonia congenita (Thomsen's disease) (Steinmeyer *et al.* 1994). The dominant nature of this inherited disease was explained by a multimeric structure of the channel. We suggested that dominant mutated subunits can still associate with the normal ones (encoded by the other allele in heterozygous patients) and thereby lead to their inactivation (dominant negative effect). The degree of this inhibition should depend on the stoichiometry of the channel complex, and the effect one mutated subunit has in this complex. Steinmeyer *et al.* (1994) analysed two human mutations differing in the extent of the dominant negative effect. We could show that the less deleterious mutation still leads to functional channels when only one mutated subunit is incorporated into the complex. However, these channels differ in kinetics and anion selectivity. Incorporation of more mutated subunits led to a loss of channel activity. Titration analysis strongly suggested that functional ClC-1 channels have at least three subunits (Steinmeyer *et al.* 1994). For symmetry reasons, and taking into account the double-barreled structure of the ClC-0 channel, we think that functional ClC channels are tetramers. However, a recent study on solubilized, immunopurified ClC-0 protein suggests that ClC-0 is a dimer (Middleton *et al.* 1994). This discrepancy remains unresolved.

Functional properties of ClC channels

All ClC channels which can be unambiguously expressed (ClC-0, ClC-1 and ClC-2) have a high chloride selectivity (with a halide sequence of Cl > Br > I) and show a voltage-dependent block by iodide to various degrees. Another common feature is a rather small single channel conductance. It is largest for the *Torpedo* channel ClC-0 (about 9 pS). This is large enough to be resolved by single-channel recordings (Miller & White, 1984; Bauer *et al.* 1991) which revealed the typical 'double-barrelled' structure of this channel. The conductance of the major skeletal muscle chloride channel ClC-1 is still smaller (slightly below 1 pS), which could only be determined by non-stationary noise analysis (Pusch *et al.* 1994). Unfortunately, this largely eliminates the possibility to answer the important question of whether ClC-1 is a 'double-barrelled' channel as well. Also by noise analysis, ClC-2 was found to have a single-channel conductance of 3–5 pS (M. Pusch & T.J. Jentsch, unpublished).

ClC-0, ClC-1 and ClC-2 all have very characteristic voltage and time dependencies of their gating behaviour. ClC-0 has a slow gate which operates on both pores of the 'double-barrelled' channel and opens it upon hyperpolarization, and a fast gate which operates on single 'protochannels', opening these with an opposite voltage dependence (upon depolarization) (Miller & White, 1984; Jentsch *et al.* 1990; Bauer *et al.* 1991). ClC-1 lacks a slow gate, but also has a fast gate which opens the channel with depolarization (or, conversely, closes it upon hyperpolarization) (Steinmeyer *et al.* 1991*a*; Pusch *et al.* 1994). It also shows a typical inward-rectification in the positive voltage range. ClC-2 has a slow gate, which opens the channel upon very strong, unphysiological hyperpolarization within several seconds, but lacks a fast gate (Gründer *et al.* 1992; Thiemann *et al.* 1992).

The physiological function for both ClC-0 and ClC-1 is to

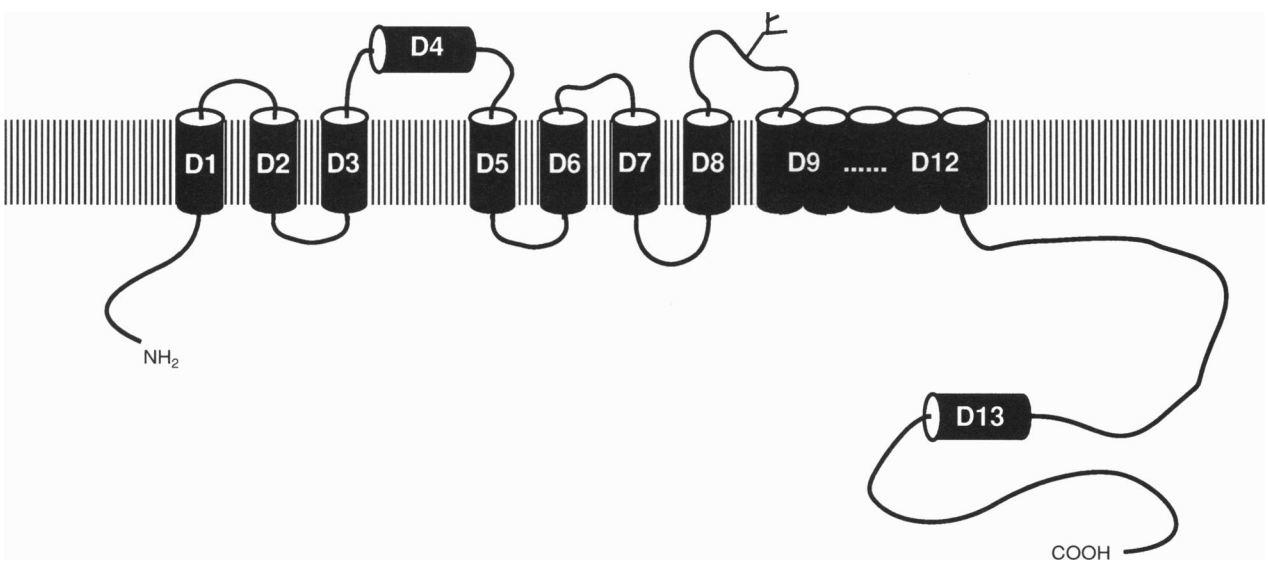

Fig. 3. Preliminary transmembrane model for ClC proteins. It is based mainly on hydropathy analysis (Jentsch *et al.* 1990). Evidence for the cytoplasmic localization of D13 comes from transplantation studies (Gründer *et al.* 1992), and for the extracellular localization of the D8–D9 linker from glycosylation studies (Kieferle *et al.* 1994). Based on low hydropathy in the D4 region, and low conservation between different ClC proteins, we hypothesize that it does not cross the membrane. The topology of the D9–D12 region is unknown.

stabilize the plasma membrane voltage, which is close to the chloride equilibrium potential in these cells. In the electric organ of *Torpedo*, this is necessary for the generation of large currents by keeping the non-innervated surface highly conductive at a constant potential while the innervated side is being depolarized. In skeletal muscle, ClC-1 activity is essential to ensure electrical stability of this excitable membrane. This is best illustrated by the fact that several myotonic diseases are due to mutations in this channel. Thus in myotonic ADR mice ClC-1 is functionally destroyed by the insertion of a transposon (Steinmeyer *et al.* 1991*b*), while point mutations in ClC-1 are the cause for myotonia in other mouse strains (Gronemeier *et al.* 1994). In humans, both recessive and dominant forms of myotonia (Becker type and Thomsen type, respectively) are caused by mutations in ClC-1 (Koch *et al.* 1992; George *et al.* 1993; Heine *et al.* 1994; Lorenz *et al.* 1994; Meyer-Kleine *et al.* 1994; Steinmeyer *et al.* 1994). As mentioned above, a detailed analysis of dominant human mutations came to the conclusion that ClC-1 functions as a multimer, most likely a tetramer (Steinmeyer *et al.* 1994).

While ClC-2 can be activated by unphysiologically strong hyperpolarization, its natural mechanism of activation is probably cell swelling. Oocyte-expressed ClC-2 can be activated reversibly by extracellular hypotonicity (Gründer *et al.* 1992). Thus the ubiquitous ClC-2 probably contributes to regulatory volume decrease in a large variety of cells and tissues. A domain of about 20 amino acids at the cytoplasmic amino terminus of the protein is absolutely required for its activation by swelling or by hyperpolarization. Extensive mutagenesis showed that some amino acids within this domain can be replaced without destroying its function (Gründer *et al.* 1992). So far we could not find any mutation which dissociates swelling activation from voltage activation. As this N-terminal inactivation domain could be transplanted to the C-terminus without loss of function, we envisage a model somewhat similar to the 'ball-and-chain' model for the inactivation of potassium channels. We postulate that this domain binds to a receptor at the channel backbone, thereby leading to a closure of the channel. We hypothesize that hyperpolarization or cell swelling leads to a decrease in the affinity of this receptor, indirectly opening the channel. The characterization of this putative receptor is in progress.

We were unable to functionally express many of the newer members of this gene family (for the kidney channels see Kieferle *et al.* 1994), although other members of this gene family reliably yielded large currents (in excess of 10 μA) in the same batches of oocytes. With the kidney channels, we not only replaced the 5′ and 3′ untranslated regions of these channels (Kieferle *et al.* 1994) (a manoeuvre successful for boosting expression of other channels, including several ClC channels), but also resorted to constructing chimeric channels having the N- or C-terminus of the channels replaced by the corresponding segments of ClC-0. However, none of these (and many other) tricks proved to be successful. We tried similar strategies with ClC-3 and ClC-4, again without success. We also co-expressed these channels in several different combinations. This is in contrast to the work of Sasaki, Marumo and co-workers, who published currents for ClC-K1 (Uchida *et al.* 1993), ClC-3 (Kawasaki *et al.* 1994), ClC-K2 (Adachi *et al.* 1994), and surprisingly even for a splice variant of ClC-K2 lacking the second putative transmembrane domain (D2), including some highly conserved sequences. In all cases, these workers detected very similar time-independent outwardly rectifying chloride currents with a rather high iodide permeability. We also observe similar currents in some batches of oocytes, but find these currents also in uninjected controls or oocytes injected with other (non-functional) RNAs. To verify the importance of D2 (deleted in the ClC-K2 splice variant of Adachi *et al.* 1994), we deleted it in ClC-0; this destroyed its channel activity (S. Kieferle & T.J. Jentsch, unpublished). Thus we think it is safer to classify these ClC members as putative channels until their function has been established unambiguously.

In conclusion, the ClC family of voltage-gated chloride channels is a large gene family whose members are likely to have different functions in various tissues and cells. The physiological functions are clearest for ClC-1 (control of muscle excitability) and ClC-2 (cell volume control), but is still obscure for new family members which cannot yet be reliably expressed as chloride channels. Site-directed mutagenesis has already yielded important insights into the structure and function of ClC-1 and ClC-2, and will soon give us information on the basic structures involved in permeation and gating of these new channels.

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